METHOD OF DETERMINING DIHYDROPYRIMIDINE DEHYDROGENASE GENE EXPRESSION

FIELD OF THE INVENTION

[001] The present invention relates to prognostic methods which are useful in medicine, particularly cancer chemotherapy. The invention also relates to assessment of gene expression of tumor cells of a patient. More specifically, the invention relates to oligonucleotides and methods comprising their use for detecting levels of Dihydropyrimidine dehydrogenase (*DPD*) mRNA expression using RT-PCR.

BACKGROUND OF THE INVENTION

[002] Cancer arises when a normal cell undergoes neoplastic transformation and becomes a malignant cell. Transformed (malignant) cells escape normal physiologic controls specifying cell phenotype and restraining cell proliferation. Transformed cells in an individual's body thus proliferate in the absence of these normal controls, thus forming a tumor.

[003] When a tumor is found, the clinical objective is to destroy malignant cells selectively while mitigating any harm caused to normal cells in the individual undergoing treatment. Chemotherapy is based on the use of drugs that are selectively toxic (cytotoxic) to cancer cells. Several general classes of chemotherapeutic drugs have been developed, including drugs that interfere with nucleic acid synthesis, protein synthesis, and other vital metabolic processes.

[004] 5-Fluorouracil (5-FU) is a very widely used drug for the treatment of many different types of cancers, including major cancers such as those of the GI tract and breast (Moertel, C.G. New Engl. J. Med., *330*:1136-1142, 1994). For more than 40 years the standard first-line treatment for colorectal cancer was the use of 5-FU alone, but it was supplanted as "standard of care" by the combination of 5-FU and CPT-11 (Saltz *et al.*, Irinotecan Study Group. New England Journal of Medicine. 343:905-14, 2000). Recently, the combination of 5-FU and oxaliplatin has produced high response rates in colorectal cancers (Raymond *et al.*, Semin. Oncol., 25:4-12, 1998). Thus, it is likely that 5-FU will be used in cancer treatment for many years because it remains the central component of current chemotherapeutic regimens. In addition, single agent 5-FU therapy continues to be used for patients in whom combination therapy with CPT-11 or oxaliplatin is likely to be excessively toxic.

[005] 5-FU is typical of most anti-cancer drugs in that only a minority of patients experience a favorable response to the therapy. Large randomized clinical trials have shown the overall response rates of tumors to 5-FU as a single agent for patients with metastatic colorectal cancer to be in the 15-20% range (Moertel, C.G. New Engl. J. Med., *330*:1136-1142, 1994). In combination with other chemotherapeutics mentioned above, tumor response rates to 5-FU-based regimens have been increased to almost 40%. Nevertheless, the majority of treated patients derive no tangible benefit from having received 5-FU based chemotherapy, and are subjected to significant risk, discomfort, and expense. Since there has been no reliable means of anticipating the responsiveness of an individual's tumor prior to treatment, the standard clinical practice has been to subject all patients to 5-FU-based treatments, fully recognizing that the majority will suffer an unsatisfactory outcome.

[006] The mechanism of action and the metabolic pathway of 5-FU have been intensively studied over the years to identify the most important biochemical determinants of the drug's anti-tumor activity. The ultimate goal was to improve the clinical efficacy of 5-FU by a) modulation of its intracellular metabolism and biochemistry and b) measuring response determinants in patients' tumors prior to therapy to predict which patients are most likely to respond (or not to respond) to the drug. Two major determinants emerged from these studies: 1) the identity of the target enzyme of 5-FU, thymidylate synthase (*TS*) and 2) the identity of the 5-FU catabolic enzyme, dihydropyrimidine dehydrogenase (*DPD*).

[007] The first studies in the area of tumor response prediction to 5-FU based therapy centered on the target enzyme TS in colorectal cancer. Leichman *et al* (Leichman *et al.*, ,J. Clin Oncol., *15*:3223-3229, 1997) carried out a prospective clinical trial to correlate tumor response to 5-FU with *TS* gene expression as determined by RT-PCR in pre-treatment biopsies from colorectal cancers. This study showed: 1) a large 50-fold range of *TS* gene expression levels among these tumors, and 2) strikingly different levels of *TS* gene expression between responding and non-responding tumors. The range of *TS* levels of the responding groups (0.5-4.1, relative to an internal control) was narrower than that of the non-responding groups (1.6-23.0, relative to an internal control). The investigators determined a resulting "non-response cutoff" threshold level of *TS* expression above which there were only non-responders. Thus, patients with *TS* expression above this "non-response cutoff" threshold could be positively identified as non-responders prior to therapy. The "no response" classification included all therapeutic responses with <50% tumor shrinkage, progressing growth resulting in a >25% tumor increase and non-progressing tumors with either <50% shrinkage, no change or <25% increase.

These tumors had the highest *TS* levels. Thus, high *TS* expression identifies particularly resistant tumors. *TS* expression levels above a certain threshold identified a subset of tumors not responding to 5-FU, whereas *TS* expression levels below this number predicted an appreciably higher response rate yet did not specifically identify responding tumors.

[008] Subsequent studies investigated the usefulness of *DPD* expression levels as a tumor response determinant to 5-FU treatment in conjunction with *TS* expression levels. DPD is a catabolic enzyme which reduces the 5,6 double bond of 5-FU, rendering it inactive as a cytotoxic agent. Previous studies have shown that DPD levels in normal tissues could influence the bio-availability of 5-FU, thereby modulating its pharmacokinetics and anti-tumor activity (Harris *et al.*, Cancer Res., *50*: 197-201, 1990). Additionally, evidence has been presented that DPD levels in tumors are associated with sensitivity to 5-FU (Etienne *et al.*, J. Clin. Oncol., 13: 1663-1670, 1995; Beck *et al.*, Eur. J. Cancer, 30: 1517-1522, 1994). Salonga *et al*, (Clin Cancer Res., 6:1322-1327, 2000) investigated gene expression of *DPD* as a tumor response determinant for 5-FU/leucovorin treatment in a set of tumors in which *TS* expression had already been determined. As with *TS*, the range of *DPD* expression among the responding tumors was relatively narrow (0.6- 2.5, 4.2-fold; relative to an internal control) compared with that of the non-responding tumors (0.2-16, 80-fold; relative to an internal control). There were no responding tumors with a *DPD* expression greater than a threshold level of about 2.5. Furthermore, *DPD* and *TS* expression levels showed no correlation with one another, indicating that they are independently regulated genes. Among the group of tumors having both *TS* and *DPD* expression levels below their respective "non-response cut-off"

threshold levels, 92% responded to 5-FU/LV. Thus, responding tumors could be identified on the basis of low expression levels of *DPD* and *TS*.

[009] *DPD* is also an important marker for 5-FU toxicity. It was observed that patients with very low *DPD* levels (such as in DPD Deficiency Syndrome; i.e. thymine uraciluria) undergoing 5-FU based therapy suffered from life-threatening toxicity (Lyss *et al.*, Cancer Invest., 11: 2390240, 1993). Indeed, the importance of DPD levels in 5-FU therapy was dramatically illustrated by the occurrence of 19 deaths in Japan from an unfavorable drug interaction between 5-FU and an anti-viral compound, Sorivudine (Diasio *et al.*, Br. J. Clin. Pharmacol. 46, 1-4, 1998). It was subsequently discovered that a metabolite of Sorivudine is a potent inhibitor of DPD. This treatment resulted in DPD Deficiency Syndrome-like depressed levels of DPD which increased the toxicity of 5-FU to the patients (Diasio *et al.*, Br. J. Clin. Pharmacol. 46, 1-4, 1998).

[010] Thus, because of a) the widespread use of 5-FU protocols in cancer treatment, b) the important role of *DPD* expression in predicting tumor response to 5-FU and c) the sensitivity of individuals with DPD-Deficiency Syndrome to common 5-FU based treatments, it is clear that accurate determination of *DPD* expression levels prior to chemotherapy will provide an important benefit to cancer patients.

[011] Measuring DPD enzyme activity requires a significant amount of fresh tissue that contains active enzyme. Unfortunately, most pre-treatment tumor biopsies are available only as fixed paraffin embedded (FPE) tissues, particularly formalin-fixed parafin embedded tissues which do not contain active enzyme. Moreover, biopsies generally contain only a very small amount of heterogeneous tissue.

[012] RT-PCR primer and probe sequences are available to analyze *DPD* expression in frozen tissue or fresh tissue. However, those primers are unsuitable for the quantification of *DPD* mRNA from fixed tissue by RT-PCR. Heretofore, existing primers give no or erratic results. This is thought to be due to the a) inherently low levels of *DPD* RNA; b) very small amount of tissue embedded in the paraffin; and c) degradation of RNA in the paraffin into short pieces of <100 bp. As a result, other investigators have made a concerted, yet unsuccessful efforts to obtain oligonucleotide primer sets allowing for such a quantification of *DPD* expression in paraffinized tissue. Thus, there is a need for method of quantifying *DPD* mRNA from fixed tissue in order to provide an early prognosis for proposed cancer therapies. Because it has been shown that DPD enzyme activity and corresponding mRNA expression levels are well correlated (Ishikawa *et al.*, Clin. Cancer Res., 5:883-889, 1999; Johnson *et al.*, Analyt. Biochem. 278: 175-184, 2000), measuring *DPD* mRNA expression in FPE specimens provides a way to assess the *DPD* expression levels status of patients without having to determine enzyme activity in fresh tissues. Furthermore, FPE specimens are readily amenable to microdissection, so that *DPD* gene expression can be determined in tumor tissue uncontaminated with stromal tissue.

[013] Accordingly, it is the object of the invention to provide a method for assessing *DPD* levels in tissues and prognosticate the probable resistance of a patient's tumor to treatment with 5-FU based therapies, by determining the amount of *DPD* mRNA in a patient's tumor cells and comparing it to a predetermined threshold expression level.

SUMMARY OF THE INVENTION

[014] In one aspect of the invention there are provided oligonucleotide primers having the sequence of DPD3A-51F (SEQ ID NO: 1) or DPD3A-134R (SEQ ID NO:2), as well as oligionucleotide primers DPD3b-651F (SEQ ID NO: 7) and DPD3b-736R (SEQ ID NO: 8) and sequences substantially identical thereto. The invention also provides for oligonucleotide primers having a sequence that hybridizes with DPD3A-51F (SEQ ID NO: 1), DPD3A-134R (SEQ ID NO:2), DPD3b-651F (SEQ ID NO:7), DPD3b-736R (SEQ ID NO: 8) or complements thereof under stringent conditions.

[015] Moreover, this invention relates to a method for determining a chemotherapeutic regimen, comprising obtaining an mRNA sample from a tumor specimen; determining *DPD* gene expression level in the specimen; comparing the determined *DPD* gene expression levels with a predetermined threshold level for that gene; and determining a chemotherapeutic regimen based on the results of the comparison of the determined gene expression level with the predetermined threshold level.

[016] The invention further relates to a method of normalizing the uncorrected gene expression (UGE) of *DPD* relative to an internal control gene in a tissue sample analyzed using Taqman technology to previously published *DPD* expression levels relative to an internal control.

BRIEF DESCRIPTION OF THE DRAWINGS

[017] **Figure 1** is a graph showing a comparison of four different oligionucleotide primer pairs for their ability to amplify *DPD* mRNA derived from 10 different formalin-FPE tissue samples. Samples #1-5, and #8-10 are derived from colon

tumor, #6 from bronchoalveolar tumor and #7 from small bowel tumor biopsies. Oligonucleotide primer pairs DPD1 (DPD-70F, (SEQ ID NO: 3) and DPD-201R, (SEQ ID NO: 4)), DPD2 (DPD2p-1129F (SEQ ID NO: 5) and DPD2p-1208R (SEQ ID NO: 6)) are not effective in measuring *DPD* mRNA levels in these samples. Oligonucleotide primer pairs DPD3A (DPD3a-51F (SEQ ID NO: 1) and DPD3a-134R (SEQ ID NO: 2)) and DPD3B (DPD3b-651F (SEQ ID NO: 7) and DPD3b-736R (SEQ ID NO: 8)) are effective in ascertaining *DPD* levels in various samples.

[018] **Figure 2** is a graph showing a comparison of *DPD* mRNA amplification efficiency of the oligonucleotide primer pairs DPD3A (DPD3a-51F (SEQ ID NO: 1) and DPD3a-134R (SEQ ID NO: 2)) and DPD1 (DPD-70F (SEQ ID NO: 3) and DPD-201R (SEQ ID NO: 4)) in frozen tissue samples. The graph illustrates that not only is the oligonucleotide primer pair DPD3A (DPD3a-51F (SEQ ID NO: 1) and DPD3a-134R (SEQ ID NO: 2)) also effective in measuring *DPD* expression levels in frozen tissue samples (as well as FPE derived samples) it is more efficient than the oligonucleotide primer pair DPD1 (DPD-70F (SEQ ID NO: 3) and DPD-201R (SEQ ID NO: 4)).

[019] **Figure 3** is a chart illustrating how to calculate *DPD* expression relative to an internal control gene. The chart contains data obtained with two test samples, (unknowns 1 and 2), and illustrates how to determine the uncorrected gene expression data (UGE) UCG. The chart also illustrates how to normalize UGE generated by the Taqman instrument with previously published *DPD* values. This is accomplished by multiplying UGE to a correction factor K_{DPD}. The internal control gene in the figure is β-actin and the calibrator RNA is Universal PE RNA; Cat

#4307281, lot # 3617812014 from Applied Biosystems.

DETAILED DESCRIPTION OF THE INVENTION

[020] The present inventors disclose oligonucleotide primers and oligonucleotide primers substantially identical thereto that allow accurate assessment of *DPD* expression in tissues. These oligonucleotide primers, DPD3a-51F (SEQ ID NO: 1) and DPD3a-134R (SEQ ID NO: 2), (also referred to herein as the oligonucleotide primer pair DPD3A) and oligionucleotide primers DPD3b-651F (SEQ ID NO: 7) and DPD3b-736R (SEQ ID NO: 8), (also referred to herein as the oligonucleotide primer pair DPD3B) are particularly effective when used to measure *DPD* gene expression in fixed paraffin embedded (FPE) tumor specimens.

[021] "Substantially identical" in the nucleic acid context as used herein, means that the oligonucleotides hybridize to a target under stringent conditions, and also that the nucleic acid segments, or their complementary strands, when compared, are the same when properly aligned, with the appropriate nucleotide insertions and deletions, in at least about 60% of the nucleotides, typically, at least about 70%, more typically, at least about 80%, usually, at least about 90%, and more usually, at least, about 95-98% of the nucleotides. Selective hybridization exists when the hybridization is more selective than total lack of specificity. See, Kanehisa, Nucleic Acids Res., 12:203-213 (1984).

[022] This invention includes substantially identical oligonucleotides that hybridize under stringent conditions (as defined herein) to all or a portion of the oligonucleotide primer sequence of DPD3A-51F (SEQ ID NO:1), its complement, DPD3A-134R (SEQ ID NO: 2) or its complement. Furthermore, this invention also

includes substantially identical oligonucleotides that hybridize under stringent conditions (as defined herein) to all or a portion of the oligonucleotide primer sequence DPD3b-651F (SEQ ID NO: 7) its complement, DPD3b-736R (SEQ ID NO: 8), or its complement.

[023] Under stringent hybridization conditions, only highly complementary, i.e., substantially identical nucleic acid sequences hybridize. Preferably, such conditions prevent hybridization of nucleic acids having 4 or more mismatches out of 20 contiguous nucleotides, more preferably 2 or more mismatches out of 20 contiguous nucleotides, most preferably one or more mismatch out of 20 contiguous nucleotides.

[024] The hybridizing portion of the nucleic acids is typically at least 10 (e.g., 15) nucleotides in length. The hybridizing portion of the hybridizing nucleic acid is at least about 80%, preferably at least about 95%, or most preferably about at least 98%, identical to the sequence of a portion or all of oligonucleotide primer DPD3A-51F (SEQ ID NO:1), its complement, DPD3A-134R (SEQ ID NO: 2) or its complement. Additionally, the hybridizing portion of the hybridizing nucleic acid is at least least about 80%, preferably at least about 95%, or most preferably about at least 98%, identical to the sequence of a portion or all of oligonucleotide primer DPD3b-651F (SEQ ID NO: 7), its complement, DPD3b-736R (SEQ ID NO: 8) or its complement.

[025] Hybridization of the oligonucleotide primer to a nucleic acid sample under stringent conditions is defined below. Nucleic acid duplex or hybrid stability is expressed as a melting temperature (T_m), which is the temperture at which the probe dissociates from the target DNA. This melting temperature is used to define the

required stringency conditions. If sequences are to be identified that are substantially identical to the probe, rather than identical, then it is useful to first establish the lowest temperature at which only holmologous hybridization occurs with a particular concentration of salt (e.g. SSC or SSPE). Then assuming that 1% mismatching results in a 1^0C decrease in T_m, the temperatre of the final wash in the hybridization reaction is reduced accordingly (for example, if sequences having >95% identity with the probe are sought, the final wash temperature is decrease by 5^0 C). In practice, the change in T_m can be between 0.5^0C and 1.5^0C per 1% mismatch.

[026] Stringent conditions involve hybridizing at 68^0 C in 5x SSC/5x Denhart's solution/1.0% SDS, and washing in 0.2x SSC/0.1% SDS at room temperature. Moderately stringent conditions include washing in 3x SSC at 42^0 C. The parameters of salt concentration and temperature may be varied to achieve optimal level of identity between the primer and the target nucleic acid. Additional guidance regarding such conditions is readily available in the art, for example, Sambrook, Fischer and Maniatis, Molecular Cloning, a laboratory manual, (2nd ed.), Cold Spring Harbor Laboratory Press, New York, (1989) and F. M. Ausubel et al eds., Current Protocols in Molecular Biology, John Wiley and Sons (1994).

[027] This aspect of the invention involves use of a method for reliable extraction of RNA from an FPE specimen and second, determination of the content of *DPD* mRNA in the specimen by using oligonucleotide primers oligionucleotide primer pair DPD3A (DPD3a-51F (SEQ ID NO: 1) and DPD3a-134R (SEQ ID NO: 2)) or oligonucleotides substantially identical thereto or DPD3B (DPD3b-651F (SEQ ID NO: 7) and DPD3b-736R (SEQ ID NO: 8)) or oligonucleotides substantially

identical thereto, for carrying out reverse transcriptase polymerase chain reaction. RNA is extracted from the FPE cells by any of the methods for mRNA isolation from such samples as described in US Patent Application No. 09/469,338, filed December 20, 1999, and is hereby incorporated by reference in its entirety.

[028] The oligonucleotide primers of the invention enable accurate assessment of *DPD* expression in a fixed paraffin embedded (FPE) tissue. (Figure 1) Additionally, the oligonucleotide primers of the present invention are accurate for determining *DPD* expression levels in fresh or frozen tissue, i.e. they have high specificity for their target RNA. Thus, methods of the invention are not limited to use of paraffin embedded tissue. Oligonucleotide primers disclosed herein are capable of enabling accurate assessment of *DPD* gene expression in a fixed paraffin embedded tissue, as well as in frozen or fresh tissue. (Figure 2). This is due to the fact that the mRNA derived from FPE samples is more fragmented relative to that of fresh or frozen tissue and is therefore, more difficult to quantify. Thus, the present invention provides oligonucleotide primers that are suitable for use in assaying *DPD* expression levels in FPE tissue, where previously there existed no suitable assay. See Figure 1.

[029] Expression of *DPD* mRNA is correlated with clinical resistance to 5-FU-based chemotherapy. In particular, expression of high levels of *DPD* mRNA correlates with resistance to 5-FU-based chemotherapies.

[030] The methods of this invention are applied over a wide range of tumor types. This allows for the preparation of individual "tumor expression profiles" whereby expression levels of *DPD* may be determined in individual patient samples and response to various chemotherapeutics can be predicted. Most preferably, the

methods of the present invention are applied to bronchalveolar, small bowel or colon tumors. For application of some embodiments of the invention to particular tumor types, it is preferable to confirm the relationship of the measurement to clinical resistance by compiling a data-set of the correlation of the particular *DPD* expression parameter measured and clinical resistance to 5-FU-based chemotherapy.

[031] The present methods can be applied to any type of tissue. For example, for examination of resistance of tumor tissue, it is desirable to examine the tumor tissue. Preferably, it is desirable to also examine a portion of normal tissue from the patient from which the tumor is obtained. Patients whose normal tissues are resistant to 5-FU-based chemotherapeutic compounds, but whose tumors are expected to be sensitive to such compounds, may then be treated with higher amounts of the chemotherapeutic composition.

[032] The methods of the present invention include the step of obtaining sample of cells from a patient's tumor. Solid or lymphoid tumors, or parts thereof are surgically resected from the patient. If it is not possible to extract RNA from the tissue sample soon after its resection, the sample may then be fixed or frozen. It will then be used to obtain RNA. RNA extracted and isolated from frozen or fresh samples of resected tissue is extracted by any method known in the art, for example, Sambrook, Fischer and Maniatis, Molecular Cloning, a laboratory manual, (2nd ed.), Cold Spring Harbor Laboratory Press, New York, (1989). Preferably, care is taken to avoid degradation of RNA during the extraction process.

[033] Alternatively, tissue obtained from the patient may be fixed, preferably by formalin (formaldehyde) or gluteraldehyde treatment, for example. Biological samples fixed by alcohol immersion are also contemplated in the present invention.

Fixed biological samples are often dehydrated and embedded in paraffin or other solid supports known to those of skill in the art. Such solid supports are envisioned to be removable with organic solvents, allowing for subsequent rehydration of preserved tissue. Fixed and paraffin-embedded (FPE) tissue specimen as described herein refers to storable or archival tissue samples.

[034] RNA is extracted from the FPE cells by any of the methods as described in US Patent Application No. 09/469,338, filed December 20, 1999, which is hereby incorporated by reference in its entirety. Most preferably, RNA is extracted from tumor cells from a formalin-fixed and paraffin-embedded tissue specimen.

[035] In an embodiment of the invention, RNA is isolated from an archival pathological sample or biopsy which is first deparaffinized. An exemplary deparaffinization method involves washing the paraffinized sample with an organic solvent, such as xylene. Deparaffinized samples can be rehydrated with an aqueous solution of a lower alcohol. Suitable lower alcohols, for example include, methanol, ethanol, propanols, and butanols. Deparaffinized samples may be rehydrated with successive washes with lower alcoholic solutions of decreasing concentration. Alternatively, the sample is simultaneously deparaffinized and rehydrated.

[036] Once the sample is reyhdrated, RNA is extracted from the rehydrated tissue. Deparaffinized samples can be homogenized using mechanical, sonic or other means of homogenization. In one embodiment, rehydrated samples are homogenized in a solution comprising a chaotropic agent, such as guanidinium thiocyanate (also sold as guanidinium isothiocyanate).

[037] An "effective concentration of chaotropic agent" is chosen such that RNA is purified from a paraffin-embedded sample in an amount of greater than about 10

fold that isolated in the absence of a chaotropic agent. Chaotropic agents include but not limited to: guanidinium compounds, urea, formamide, potassium iodiode, potassium thiocyantate and similar compounds. The preferred chaotropic agent for the methods of the invention is a guanidinium compound, such as guanidinium isothiocyanate (also sold as guanidinium thiocyanate) and guanidinium hydrochloride. Many anionic counterions are useful, and one of skill in the art can prepare many guanidinium salts with such appropriate anions. The effective concentration of guanidinium solution used in the invention generally has a concentration in the range of about 1 to about 5M with a preferred value of about 4M. If RNA is already in solution, the guanidinium solution may be of higher concentration such that the final concentration achieved in the sample is in the range of about 1 to about 5M. The guanidinium solution also is preferably buffered to a pH of about 3 to about 6, more preferably about 4, with a suitable biochemical buffer such as Tris-Cl. The chaotropic solution may also contain reducing agents, such as dithiothreitol (DTT), (β-mercaptoethanol; BME); and combinations thereof. The chaotropic solution may also contain RNAse inhibitors.

[038] Homogenized samples may be heated to a temperature in the range of from about 50 to about 100 °C in a chaotropic solution, containing an effective amount of a chaotropic agent, such as a guanidinium compound. A preferred chaotropic agent is guanidinium thiocyanate.

[039] RNA is then recovered from the solution by, for example, phenol chloroform extraction, ion exchange chromatography or size-exclusion chromatography. RNA may then be further purified using the techniques of extraction, electrophoresis, chromatography, precipitation or other suitable techniques.

[040] The quantification of *DPD* mRNA from purified total mRNA from fresh, frozen or fixed is preferably carried out using reverse-transcriptase polymerase chain reaction (RT-PCR) methods common in the art, for example. Other methods of quantifying of *DPD* mRNA include for example, the use of molecular beacons and other labeled probes useful in multiplex PCR. Additionally, the present invention envisages the quantification of *DPD* mRNA via use of a PCR-free systems employing, for example fluorescent labeled probes similar to those of the Invader® Assay (Third Wave Technologies, Inc.). Most preferably, quantification of *DPD* cDNA and an internal control or house keeping gene (e.g. β-actin) is done using a fluorescence based real-time detection method (ABI PRISM 7700 or 7900 Sequence Detection System [TaqMan®], Applied Biosystems, Foster City, CA.) or similar system as described by Heid *et al.*, (Genome Res 1996;6:986-994) and Gibson *et al.*(Genome Res 1996;6:995-1001). The output of the ABI 7700 (TaqMan® Instrument) is expressed in Ct's or "cycle thresholds". With the TaqMan® system, a highly expressed gene having a higher number of target molecules in a sample generates a signal with fewer PCR cycles (lower Ct) than a gene of lower relative expression with fewer target molecules (higher Ct).

[041] The present invention resides in part in the finding that the relative amount of *DPD* mRNA is correlated with resistance to the chemotherapeutic agent 5-FU. It has been found herein that tumors expressing high levels of *DPD* mRNA are likely to be resistant to 5-FU. Conversely, those tumors expressing low amounts of *DPD* mRNA are likely to be sensitive to 5-FU. A patient's expression of tumor *DPD* mRNA is judged by comparing it to a predetermined threshold expression level of expression of *DPD*.

[042] As used herein, a "house keeping" gene or "internal control" is meant to include any constitutively or globally expressed gene whose presence enables an assessment of *DPD* mRNA levels. Such an assessment comprises a determination of the overall constitutive level of gene transcription and a control for variations in RNA recovery. "House-keeping" genes or "internal controls" can include, but are not limited to the cyclophilin gene, β-actin gene, the transferrin receptor gene, GAPDH gene, and the like. Most preferably, the internal control gene is β-actin gene as described by Eads *et al.*, Cancer Research 1999; 59:2302-2306.

[043] A control for variations in RNA recovery requires the use of "calibrator RNA." The "calibrator RNA" is intended to be any available source of accurately pre-quantified control RNA. Preferably, Universal PE RNA; Cat #4307281, lot # 3617812014 from Applied Biosystems is used.

[044] "Uncorrected Gene Expression (UGE)" as used herein refers to the numeric output of *DPD* expression relative to an internal control gene generated by the TaqMan® instrument. The equation used to determine UGE is shown in Example 4, and illustrated with sample calculations in Figure 3.

[045] A further aspect of this invention provides a method to normalize uncorrected gene expression (UGE) values acquired from the Taqman instrument with previously published relative gene expression values derived from non-TaqMan® technology. Preferably, the non-TaqMan® derived relative *DPD* : β-actin expression values previously published by Salonga, *et al.*, Clinical Cancer Research, 6:1322-1327, 2000, are normalized with *DPD* UGE from a tissue sample.

[046] "Corrected Relative *DPD* Expression" as used herein refers to normalized *DPD* expression whereby UGE is multiplied with a *DPD* specific correction factor

(K_{DPD}), resulting in a value that can be compared to a previously published range of values. Figure 3 and Figure 4 illustrate these calculations in detail.

[047] "Previously published" relative gene expression results are based on the ratio of the RT-PCR signal of a target gene to a constitutively expressed gene (β-Actin). In pre-TaqMan® technology studies, PCR reactions were run for a fixed number of cycles (i.e., 30) and endpoint values were reported for each sample. These values were then reported as a ratio of *DPD* expression to β-actin expression. Salonga, *et al.*, Clinical Cancer Research, 6:1322-1327, 2000.

[048] A "predetermined threshold" level of relative *DPD* expression, as defined herein, is a level of *DPD* expression above which it has been found that tumors are likely to be resistant to 5-FU. Expression levels below this threshold level are likely to be found in tumors sensitive to 5-FU. The range of relative *DPD* expression, among tumors responding to a 5-FU based chemotherapeutic regimen responding tumors is less than about 0.6 to about 2.5, (about a 4.2-fold range). Tumors not responding to a 5-FU based chemotherapeutic regimen have relative *DPD* expression of about 0.2 to about16 (about an 80-fold range). Tumors generally do not respond to 5-FU treatment if there is a relative *DPD* expression greater than about 2.0, preferably greater than about 2.5. These numerical values allow the determination of whether or not the "Corrected Relative *DPD* Expression" of a particular sample falls above or below the "predetermined threshold" level.

[049] A threshold level of Corrected Relative *DPD* Expression level of about 2.0 to about 2.5. substantially equivalent to the threshold level.

[050] The methods of the invention are applicable to a wide range of tissue and tumor types and so can be used for assessment of treatment in a patient and as a

diagnostic or prognostic tool in a range of cancers including breast, head and neck, lung, esophageal, colorectal, and others. Preferably, the present methods are applied to prognosis of bronchoalveolar, small bowel, or colon cancer.

[051] From the measurement of the amount of *DPD* mRNA that is expressed in the tumor, the skilled practitioner can make a prognosis concerning clinical resistance of a tumor to 5-FU-based chemotherapy. "5-FU-based chemotherapy" comprises administration of 5-FU, its derivatives, alone or with other chemotherapeutics or with a DPD inhibitor such as uracil, 5-ethynyluracil, bromovinyluracil, thymine, benzyloxybenzyluracil (BBU) or 5-chloro-2,4-dihydroxypyridine. Furthermore, it has been found that co-administration of a 5'-deoxy-cytidine derivative of the formula (I) with 5-FU or a derivative thereof significantly improves delivery of a chemotherapeutic agent selectively to tumor tissues as compared with the combination of 5-FU or a derivative thereof with a DPD inhibitor 5-ethynyluracil, and shows significantly improved antitumor activity in human cancer xenograft models.

[052] The invention being thus described, practice of the invention is illustrated by the experimental examples provided below. The skilled practitioner will realize that the materials and methods used in the illustrative examples can be modified in various ways. Such modifications are considered to fall within the scope of the present invention.

EXAMPLES

EXAMPLE 1

RNA Isolation from FPE Tissue

[053] RNA is extracted from paraffin-embedded tissue by the following general procedure.

A. Deparaffinization and hydration of sections:

[054] (1) A portion of an approximately 10 μM section is placed in a 1.5 mL plastic centrifuge tube.

[055] (2) 600 μL, of xylene are added and the mixture is shaken vigorously for about 10 minutes at room temperature (roughly 20 to 25 °C).

[056] (3) The sample is centrifuged for about 7 minutes at room temperature at the maximum speed of the bench top centrifuge (about 10-20,000 x g).

[057] (4) Steps 2 and 3 are repeated until the majority of paraffin has been dissolved. Two or more times are normally required depending on the amount of paraffin included in the original sample portion.

[058] (5) The xylene solution is removed by vigorously shaking with a lower alcohol, preferably with 100% ethanol (about 600 μL) for about 3 minutes.

[059] (6) The tube is centrifuged for about 7 minutes as in step (3). The supernatant is decanted and discarded. The pellet becomes white.

[060] (7) Steps 5 and 6 are repeated with successively more dilute ethanol solutions: first with about 95% ethanol, then with about 80% and finally with about 70% ethanol.

[061] (8) The sample is centrifuged for 7 minutes at room temperature as in step

(3). The supernatant is discarded and the pellet is allowed to dry at room temperature for about 5 minutes.

B. RNA Isolation with Phenol-Chloroform

[062] (1) 400 μL guanidine isothiocyanate solution including 0.5% sarcosine and 8 μL dithiothreitol is added.

[063] (2) The sample is then homogenized with a tissue homogenizer (Ultra-Turrax, IKA-Works, Inc., Wilmington, NC) for about 2 to 3 minutes while gradually increasing the speed from low speed (speed 1) to high speed (speed 5).

[064] (3) The sample is then heated at about 95 °C for about 5-20 minutes. It is preferable to pierce the cap of the tube containing the sample before heating with a fine gauge needle. Alternatively, the cap may be affixed with a plastic clamp or with laboratory film.

[065] (4) The sample is then extracted with 50 μL 2M sodium acetate at pH 4.0 and 600 μL of phenol/chloroform/isoamyl alcohol (10:1.93:0.036), prepared fresh by mixing 18 mL phenol with 3.6 mL of a 1:49 isoamyl alcohol:chloroform solution. The solution is shaken vigorously for about 10 seconds then cooled on ice for about 15 minutes.

[066] (5) The solution is centrifuged for about 7 minutes at maximum speed. The upper (aqueous) phase is transferred to a new tube.

[067] (6) The RNA is precipitated with about 10 μL glycogen and with 400 μL isopropanol for 30 minutes at -20 °C.

[068] (7) The RNA is pelleted by centrifugation for about 7 minutes in a benchtop centrifuge at maximum speed; the supernatant is decanted and discarded; and the pellet washed with approximately 500 μL of about 70 to 75% ethanol.

[069] (8) The sample is centrifuged again for 7 minutes at maximum speed. The supernatant is decanted and the pellet air dried. The pellet is then dissolved in an appropriate buffer for further experiments (e.g. 50 pI. 5mM Tris chloride, pH 8.0).

EXAMPLE 2

mRNA Reverse Transcription and PCR

[070] **Reverse Transcription:** RNA was isolated from microdissected or non-microdissected formalin fixed paraffin embedded (FPE) tissue as illustrated in Example 1 and as previously described in U.S. Application No. 09/469,338 filed December 20, 1999, which is hereby incorporated by reference in its entirety. After precipitation with ethanol and centrifugation, the RNA pellet was dissolved in 50 ul of 5 mM Tris/Cl at pH 8.0. The resulting RNA was reverse transcribed with random hexamers and M-MLV from Life Technologies (CAT#28025-02.). The reverse transcription was accomplished by mixing 25 µl of the RNA solution with 25.5 µl of "reverse transcription mix" (see below). The reaction was placed in a thermocycler for 8 min at 26° C (for binding the random hexamers to RNA), 45 min at 42° C (for the M-MLV reverse transcription enzymatic reaction) and 5 min at 95° C (for heat inactivation of DNAse).

[071] "Reverse transcription mix" consisted of 10 ul 5X buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgCl2), 0.5 ul random hexamers (50 O.D. dissolved in 550 ul of 10 mM Tris-HCl pH 7.5) 5 ul 10 mM dNTPs (dATP, dGTP, dCTP and dTTP), 5 ul 0.1 M DTT, 1.25 ul BSA (3mg/ml in 10 mM Tris-HCL, pH 7.5), 1.25 ul RNA Guard 24,800U/ml (RNAse inhibitor) (Porcine #27-0816, Amersham Pharmacia) and 2.5 ul MMLV 200U/ul (Life Tech Cat #28025-02).

[072] Final concentrations of reaction components were: 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl2, 1.0 mM dNTP, 1.0 mM DTT, 0.00375. mg/ml BSA, 0.62 U/ul RNA Guard and 10 U/ ul MMLV.

[073] **PCR Quantification of mRNA expression:** Quantification of *DPD* cDNA and an internal control or house keeping gene (i.e. β-actin, as described in Eads *et al.*, (Cancer Research 1999; 59:2302-2306) was done using a fluorescence based real-time detection method (ABI PRISM 7700 or 7900 Sequence Detection System [TaqMan®], Applied Biosystems, Foster City, CA.) as described by Heid *et al.*, (Genome Res 1996;6:986-994); Gibson *et al.*, (Genome Res 1996;6:995-1001). In brief, this method uses a dual labelled fluorogenic oligonucleotide probe (the TaqMan® probe) that anneals specifically within the template amplicon spanning the forward and reverse primers. Laser stimulation within the capped wells containing the reaction mixture causes emission of a 3' quencher dye (TAMRA) until the probe is cleaved by the 5' to 3' nuclease activity of the DNA polymerase during PCR extension, causing release of a 5' reporter dye (6FAM). Production of an amplicon thus causes emission of a fluorescent signal that is detected by the TaqMan®'s CCD (charge-coupled device) detection camera, and the amount of signal produced at a threshold cycle within the purely exponential phase of the PCR reaction reflecting the starting copy number of the sequence of interest. TaqMan® probe for the oligonucleotide primer pair DPD1 (DPD-70F (SEQ ID NO: 3) and DPD-201R (SEQ ID NO: 4)) is DPD-108Tc (SEQ ID NO:9). TaqMan® probe for the oligonucleotide primer pair DPD2 (DPD2p-1129F (SEQ ID NO: 5) and DPD2p-1208R (SEQ ID NO: 6)) is DPD-2p-1154Tc (SEQ ID NO: 10). TaqMan® probe for

the oligonucleotide primer pair DPD3A (DPD3a-51F (SEQ ID NO: 1) and DPD3a-134R (SEQ ID NO: 2)) is DPD3A-71Tc (SEQ ID NO: 11). TaqMan® probe for the oligonucleotide primer pair DPD3B (DPD3b-651F (SEQ ID NO: 7) and DPD3b-736R (SEQ ID NO: 8)) is DPD3b-685Tc (SEQ ID NO: 12).

[074] The PCR reaction contained olgionulceotide primers from the pair DPD1 (DPD-70F (SEQ ID NO: 3) and DPD-201R (SEQ ID NO: 4)); DPD2 (DPD2p-1129F (SEQ ID NO: 5) and DPD2p-1208R (SEQ ID NO: 6)); DPD3B (DPD3b-651F (SEQ ID NO: 7), $T_m = 58^0$ C and DPD3b-736R (SEQ ID NO: 8), $T_m = 60^0$ C); or oligonucleotide primer pair DPD3A (DPD3a-51F (SEQ ID NO: 1), $T_m = 59^0$ C and DPD3a-134R (SEQ ID NO: 2), $T_m = 59^0$ C). Each PCR reaction mixture consisted 0.5 µl of the reverse transcription reaction containing the cDNA as well as 600 nM each of both oligonucleotide primers from only one pair (DPD1, DPD2, DPD3B or DPD3A), 200 nM corresponding TaqMan® probe (for either DPD1, DPD2, DPD3B or DPD3A), 5 U AmpliTaq Gold Polymerase, 200 µM each dATP, dCTP, dGTP, 400 µM dTTP, 5.5 mM $MgCl_2$, and 1 x Taqman Buffer A containing a reference dye, to a final volume of less than or equal to 25 µl (all reagents, Applied Biosystems, Foster City, CA). Cycling conditions were, 95 °C for 10 min, followed by 45 cycles at 95 °C for 15s and 60 °C for 1 min.

EXAMPLE 3

DPD Expression in FPE Tumor Samples

[075] The oligonucleotide primer pairs DPD3A (DPD3a-51F (SEQ ID NO: 1) and DPD3a-13R (SEQ ID NO: 2)) and DPD3B (DPD3b-651F (SEQ ID NO: 7) and DPD3b-736R (SEQ ID NO: 8)) allowed robust, reproducible quantitation of *DPD*

gene expression by RT-PCR using RNA extracted from paraffin-embedded tissue. Figure 1. Oligonucleotide primer pair DPD3A (DPD3a-51F (SEQ ID NO: 1) and DPD3a-13R (SEQ ID NO: 2)) also significantly increased the sensitivity of *DPD* gene expression analysis by RT-PCR in fresh frozen tissue. Figure 2. RT-PCR was performed using the ABI Prism 7700 Sequence Detection System (Taqman®) as described in Example 2, above.

[076] Thirty cycles were used in the PCR reaction. Each cycle consisted of denaturing at 96^0 C for 1 min, annealing at 55^0 C for 1 min and extending at 72^0 C for 2 min. The amplified product using oligionucleotide primer pair DPD3A (DPD3a-51F (SEQ ID NO: 1) and DPD3a-13R (SEQ ID NO: 2)) was 84 base pairs in length. The amplified product corresponded to region of *DPD* cDNA spanning a portion of the 5' untranslated region (UTR) and running into Exon 1. The amplified product using oligionucleotide primer pair DPD3B (DPD3b-651F (SEQ ID NO: 7) and DPD3b-736R (SEQ ID NO: 8)) is 86 base pairs in length. The amplified product corresponded to amplifies a region of *DPD* cDNA corresponding to Exon 6.

[077] Oligonucleotide primer pairs DPD3A (DPD3a-51F (SEQ ID NO: 1) and DPD3a-13R (SEQ ID NO: 2)) and DPD3B (DPD3b-651F (SEQ ID NO: 7), and DPD3b-736R (SEQ ID NO: 8)) were compared to other existing primer sets for their ability to amplify *DPD* mRNA derived from 10 different FPE tissue samples. Samples #1-5, and #8-10 were derived from colon, #6 from bronchoalveolar and #7 from small bowel tumor biopsies. Other oligonucleic acid primer pairs used were DPD1 (DPD-70F (SEQ ID NO: 3) and DPD-201R (SEQ ID NO: 4)) and DPD2 (DPD2p-1129F (SEQ ID NO: 5) and DPD2p-1208R (SEQ ID NO: 6)).

[078] The oligonucleotide primer pair DPD3A (DPD3a-51F (SEQ ID NO: 1) and

DPD3a-134R (SEQ ID NO: 2)) was most effective in accurately ascertaining *DPD* levels in various samples. Oligionucleotide primer pair DPD3B (DPD3b-651F (SEQ ID NO: 7) and DPD3b-736R (SEQ ID NO: 8)) was also effective, yet did not provide as strong a signal. Results illustrated in Figure 1

EXAMPLE 4

Determining the Uncorrected Gene Expression (UGE) for DPD

[079] Two pairs of parallel reactions are carried out. The "test" reactions and the "calibration" reactions. The *DPD* amplification reaction and the β-actin internal control amplification reaction are the test reactions. A separate β-actin amplification reaction and calibrator RNA amplification reaction are the calibration reactions. The Taqman instrument will yield four different cycle threshold (Ct) values: Ct_{DPD} and $Ct_{\beta\text{-actin}}$ from the test reactions and $Ct_{calibRNA}$ and $Ct_{\beta\text{-actin}}$ from the calibration reactions.

[080] The differences in Ct values for the two reactions are determined according to the following equation:

$\Delta Ct_{test} = Ct_{DPD} - Ct_{\beta\text{-actin}}$ (From the "test" reaction)

$\Delta Ct_{calibrator} = Ct_{calibRNA} - Ct_{\beta\text{-actin}}$ (From the "calibration" reaction)

[081] Next the step involves raising the number 2 to the negative ΔCt, according to the following equations.

$2^{-\Delta Ct}_{test}$ (From the "test" reaction)

$2^{-\Delta Ct}_{calibrator}$ (From the "calibration" reaction)

[082] In order to then obtain an uncorrected gene expression for *DPD* from the Taqman instrument the following calculation is carried out:

$$\text{Uncorrected gene expression (UGE) for } DPD = 2^{-\Delta Ct}{}_{test} \; / \; 2^{-\Delta Ct}{}_{calibrator}$$

Normalizing UGE with previously published values

[083] The normalization calculation entails a multiplication of the UGE with a correction factor (K_{DPD}) specific to *DPD* and a particular calibrator RNA. The correction factor K_{DPD} can be determined using any internal control gene and any accurately pre-quantified calibrator RNA. Preferably, the internal control gene β-actin and the accurately pre-quantified calibrator RNA, Universal PE RNA; Cat #4307281, lot # 3617812014 from Applied Biosystems, are used.

[084] Normalization is accomplished using modification of the ΔCt method described by Applied Biosystems, the Taqman manufacturer, in User Bulletin #2 and described above. To carry out this procedure, the UGE of 6 different previously published test tissues was analyzed for DPD expression using the Taqman methodology described above. The internal control gene β-actin and the calibrator RNA, Universal PE RNA; Cat #4307281, lot # 3617812014 from Applied Biosystems was used.

[085] The published relative *DPD* expression level (PV) of each previously described sample A, B, C, D, E and F was divided by its corresponding Taqman derived UGE to yield an unaveraged correction factor K.

$$K_{unaveraged} = PV \; / \; UGE$$

[086] Next, all of the K values are averaged to determine a single K_{DPD} correction factor specific for *DPD*, Universal PE RNA; Cat #4307281, lot # 3617812014 calibrator RNA and β-actin.

[087] Therefore, to determine the Corrected Relative *DPD* Expression in an unknown tissue sample on a scale that is consistent with previously published pre-Taqman *DPD* expression studies, one merely multiplies the uncorrected gene expression data (UGE) derived from the Taqman apparatus with the K_{DPD} specific correction factor, given the use of the same internal control gene and calibrator RNA.

Corrected Relative *DPD* Expression = UGE x K_{DPD}

[088] A K_{DPD} may be determined using any accurately pre-quantified calibrator RNA. Future sources of accurately pre-quantified RNA can be calibrated to published samples as described in the method above or may now be calibrated against a previously calibrated calibrator RNA such as Universal PE RNA; Cat #4307281, lot # 3617812014 described above.